In Vitro Study of Phase Resetting and Phase Locking in a Time-Comparison Circuit in the Electric Fish, *Eigenmannia*

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ABSTRACT The electric fish *Eigenmannia* generates an oscillating weak electric field. The amplitude and timing information of this electric field is perceived by electroreceptors distributed on its skin. The pathway of timing information, consisting of spherical cells and giant cells, was studied in an in vitro preparation. The giant cells were identified to be endogenous oscillators and thus have the functional advantage of phase locking more easily to a periodic stimulus with a frequency in the range of the intrinsic frequency. Their spontaneous rhythmic activity was perturbed by delivering excitatory single pulses or periodic pulses via their synaptic inputs. The regular and irregular dynamics produced by periodic stimulation were discussed in the context of a mathematical analysis of the response to single pulses. Ambiguous representations of the timing of the stimulus pulse were observed and could be related to this analysis. Some spontaneously firing cells could be silenced with periodic excitatory stimulation in a narrow frequency and amplitude range. Some irregularly firing cells continued to fire periodically for several seconds after phase locking to a periodic stimulus. This study is the first description of an endogenous oscillator in a system devoted to the precise timing of sensory events.

INTRODUCTION

Phase locking is an essential feature of conveying timing in sensory systems, such as the auditory pathway in the owl (Konishi, 1991; Carr, 1993) and the electrosensory pathway in the electric fish *Eigenmannia* (Bastian and Heiligenberg, 1980; Rose and Heiligenberg, 1985; Carr et al., 1986a). Although phase locking has been studied in a variety of systems (for reviews see Glass and Mackey, 1988; Glass and Winfree, 1984), sensory systems, in which the timing is the signal to be conveyed, have been neglected. In this study, the electrosensory system of electric fish has been chosen to explore how phase locking and endogenous oscillators are employed in a sensory pathway of timing information.

In many neuronal systems, the amplitude, frequency, and phase of an endogenous oscillator (Jack et al., 1975; Jacklet, 1989; Schwindt, 1992; McCormick et al., 1992) are under the control of synaptic inputs to the oscillator. Thus, characterizing the effects of single and periodic stimuli is essential for a functional analysis (Glass and Mackey, 1988; Glass and Winfree, 1984). This approach has been applied to a variety of systems, such as pacemaker cells in the abdominal ganglia of *Aplysia* and tonic thoracic stretch receptors of *Procambarus* (Perkel et al., 1964), bursting neurons in the abdominal ganglion of *Aplysia* (Pinsker, 1977a,b), squid axon (Best, 1979; Guttman et al., 1980), cardiac pacemaker cells (Jalife and Antzelevitch, 1979; Guevara et al., 1981; Clay et al., 1984; Guevara et al., 1986; Zeng et al., 1990), Purkinje fibers (Chay and Lee, 1984), and a two-current excitable membrane model (Rinzel and Ermentrout, 1989). The electrical activity in these systems follows a limit-cycle oscillation (Jack et al., 1975), i.e., an oscillation that may be reestablished after a perturbation and can be perturbed by single stimuli delivered at different phases ϕ of the limit cycle, yielding the perturbed cycle length $T(\phi)$ as a function of the phase. This function shows either of two distinct shapes (continuous or discontinuous), depending on the stimulus magnitude (Winfree, 1977). Once $T(\phi)$ has been measured via single-pulse experiments, the response of the endogenous oscillator to a periodic stimulus with interval τ can be estimated, particularly the range of the stimulus frequency and the phase ("locking phase") at which phase locking occurs (Perkel et al., 1964; Guevara et al., 1981).

The electric fish *Eigenmannia* generates quasi-sinusoidal electric organ discharges at individually fixed frequencies (250–600 Hz) for electrolocation and communication. The electric field around the body is detected by electroreceptors located on the body surface. When two fish with similar discharge frequencies meet, each fish shifts the frequency of its electric organ discharge so as to increase the frequency difference between them ("jamming avoidance response") (for reviews see Heiligenberg, 1986, 1991; Kawasaki, 1993; Carr, 1993). The fish determines whether its neighbor has a frequency higher or lower than its own by evaluating the amplitude and the timing of the superposed electric fields on its skin (Heiligenberg et al., 1978). The timing information is conveyed to the midbrain by a chain of neuronal structures (Carr et al., 1986a,b) (Fig. 1). The afferent fibers from T-type electroreceptors terminate with electrotonic synapses onto the almost adendritic spherical cells in the electrosensory lateral line lobe. The spherical cells, in turn, send their axons through the lateral lemniscus and terminate with electrotonic synapses onto the somata of adendritic giant cells and with mixed synapses onto the dendrite of small cells in lamina 6 of the torus semicircularis in the midbrain.

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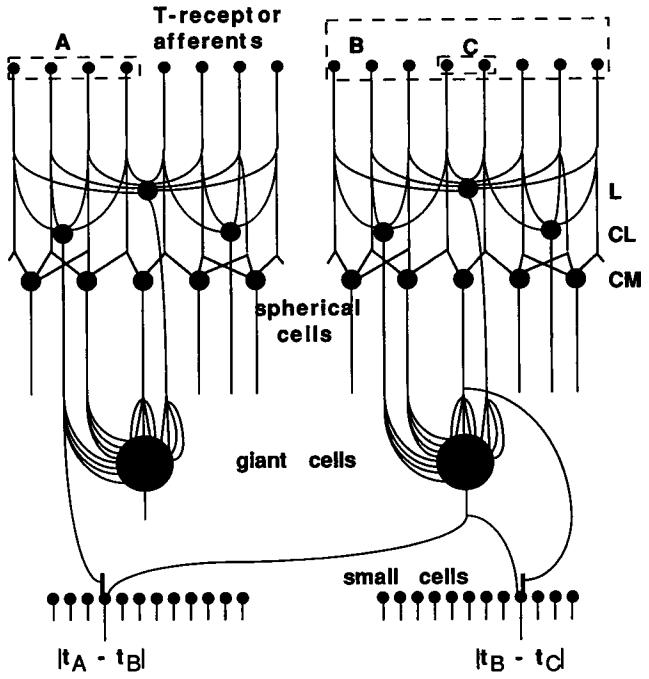

FIGURE 1 Diagrammatic representation of the time-comparison circuit. T-type electroreceptor afferents terminate with electrotonic synapses on the somata of spherical cells in the centromedial (CM), centrolateral (CL), and lateral (L) map in the electrosensory lateral line lobe. The spherical cells send axons through the lateral lemniscus and terminate with electrotonic synapses on the somata of giant cells and with mixed synapses on the dendrites of small cells in lamina 6 of the torus semicircularis. The giant cells terminate with electrotonic synapses on the somata of small cells. The timing of a spike of a spherical or giant cell represents the timing of the electric field in the receptive field of that cell. The small cells are thought to compute the difference in the timing of the spikes of their two inputs, i.e., $|t_A - t_B|$[ba] or $|t_B - t_C|$. In this figure, the relative numbers of cells and synapses represent the histological data. For clarity, only few collaterals from spherical and giant cells are included. The size of the cells is not to scale.

The giant cells terminate with electrotonic synapses onto the somata of small cells. The T-type afferents, the spherical cells, and the giant cells phase lock to the electric field in their respective receptive fields. The small cells are the first in this circuit to compare timings, i.e., the timing of the input on the dendrite with the timing of the input on the soma (Heiligenberg and Rose, 1985). Both timings have been conveyed from the skin to the small cells via phase-locked pathways.

In this paper, the pathway of timing information was studied in an in vitro preparation, and the following observations were made. The spherical cell afferents and giant cells can be maintained and stimulated in an in vitro preparation for more than 12 h. Giant cells are identified as endogenous oscillators, and the functional advantage of endogenous oscillators in this pathway is discussed. Spherical cell afferents and giant cells phase lock to periodic stimulation at certain parameter ranges. With single-stimulus pulses, the "perturbed cycle length" $T(\phi)$ was measured and the response to periodic stimulation was estimated. The timing of the stimulus pulse is only unambiguously represented by the timing of the spike, when the latency of the spike occurence after the stimulus pulse is constant. However, in some cases the latency jumped between two different values or was dependent on the frequency of the periodic stimulation. This ambiguous representation of the timing of the stimulus pulse could be related to the $T(\phi)$ curve at low stimulus amplitude. Some spontaneously firing cells could be silenced with periodic excitatory stimulation. Some irregularly firing cells continued to fire periodically for several seconds after phase locking to a periodic stimulus.

MATERIALS AND METHODS

Adult specimens of *Eigenmannia*, acquired from tropical fish dealers under the commercial name "glass knife fish" and maintained at 25°C in aquarium water, adjusted for conductivity to 20–30 kΩ·cm and pH = 7, were used in these experiments. The dissection protocol followed was similar to that of Meyer (1984), Mathiesson and Maler (1988), and Dye (1988). Each animal was cold anaesthetized in ice-cold artificial cerebrospinal fluid saline (in mM: 124 NaCl, 2 KCl, 1.25 KH_2PO_4, 1.1 $MgSO_4 \cdot 7H_2O$, 1.1 $CaCl_2 \cdot 2H_2O$, 16 $NaHCO_3$, 10 dextrose; pH set to 7.4 with $NaHCO_3$ after 30 min of bubbling with 95% O_2/5% CO_2). After the skull was opened, the cerebellum and deeper portions of the brain were removed with a suction pipette until the crossing of the lateral lemniscus was clearly visible. In the same way the tectum opticum was removed. All cranial nerves, the ganglia of the anterior lateral line nerve, and the spinal cord were severed. The remaining brain was transferred in a wide pipette to a nylon mesh and strapped down with two nylon threads, one across the forebrain and the other across the spinal cord. The mesh with the brain was then transferred into the recording chamber, which was continuously perfused with oxygenated artificial cerebrospinal fluid (see above). Moistened O_2/CO_2 was blown over the top of the chamber.

Recordings were made with quartz glass microelectrodes (Sutter) pulled on a laser puller (Sutter P2000). The electrodes were filled with 2% Neurobiotin (Vector) in 1 M KCl and beveled (Sutter BV-10) to a resistance of 30–80 MΩ. The recording electrode was positioned on the surface of the torus under visual guidance and lowered into the tissue with a manually driven manipulator (Sutter MP-85). Giant cells and spherical cell afferents were distinguished from other cells in the torus by their spontaneous activity and by their phase locking to periodic stimulation of the lateral lemniscus. Cell penetration was performed by tapping the manipulator, or "buzzing" the electrode for 1 ms. The signal, amplified through an Axoclamp 2A (Axon Instruments), was A-to-D converted (RUN Technologies, DATA PAC II) at 25 kHz sampling rate and stored on a hard disk. In some cases the data were first stored on an analog FM recorder (HP 3964A).

Stimulation was delivered with a stainless steel concentric bipolar electrode (Federick Haer and Co.) with an outer diameter of 200 μm, placed on the lateral lemniscus at the point where the two bundles cross the midline. The electrode was connected through an optically coupled stimulation isolator (WPI A350DA) to a stimulus generator (WPI 302-I). Pulses were typically 100 μs long and of 3 mA amplitude and were always set to a level well above threshold. The axons in the lateral lemniscus are heavily mylenated and, at the stimulation site, the fiber is about 400 μm thick. The large stimulus amplitude was necessary for homogeneous stimulation of all axons in the fiber, which is required for synchronized stimulation. At this current gas bubbles form at the tip of the electrode, and as a result, the brain may move slightly. The effective stimulus amplitude depends on the location of the stimulus electrode with respect to the axon that is being stimulated and thus is difficult to quantify. Direct activation of the giant cells can be excluded, because lifting the stimulus electrode slightly above the fiber (but still in the saline) abolished any response of the giant cells to the stimulus.

For intracellular labeling, cells were injected with Neurobiotin by passing 1 nA depolarizing DC current through the electrode for about 5 min. The brain was immersion fixed in 4% paraformaldehyde in 0.1 M phosphate buffer solution for more than 2 days. After vibratome sectioning at 50 μm thickness, the slices were processed according to a protocol modified from Horikawa and Armstrong (1988) (Kawasaki, 1994) to visualize Neurobiotin.

RESULTS

Lamina 6 in vitro

Spherical cell afferents and giant cells stay alive in the in vitro preparation for more than 12 h. In a healthy in vitro preparation, the electrode generally encountered at least one active giant cell axon or spherical cell axon, identified by their unique response to a stimulus pulse, each time it traveled through lamina 6. Resting membrane potentials up to -70 mV and spikes up to 80 mV were recorded. Because there are only about 380 giant cell somata, each with a diameter of 20–40 μm (Carr et al., 1986b), their encounter was much less likely and could be identified by the occurrence of membrane potential oscillations. The spontaneous and driven activity of spherical cell afferents and giant cells in this in vitro preparation is comparable to that of in vivo recordings of lamina 6 (Carr et al., 1986a).

Giant cells are endogenous oscillators

The spherical cells with somata in the electrosensory lateral line lobe send axons through the lateral lemniscus and synapse on giant cell somata and small cell dendrites in lamina 6 (Fig. 1). In the intact fish, T-type electroreceptor afferents synapse onto more than one spherical cell soma, and each spherical cell receives inputs from more than one T-type electroreceptor afferent. In the intact fish with an electric field, T-type electroreceptor afferents phase lock to the electric field, and spherical cells with overlapping receptive fields may show cross-correlation in their firing pattern due to correlated inputs. However, there are no lateral connections between spherical cells and thus, in the absence of an electric field, or after T-receptor afferents have been severed, as in the in vitro preparation, we do not expect any cross-correlation in the firing pattern of spherical cells. In the in vitro preparation the giant cells typically receive independent and irregular spike trains from three or four spherical cells (Carr et al., 1986a). However, in a healthy in vitro preparation we encountered many giant cells that fired periodically with little jitter, with a frequency close to the electric organ discharge frequency of the fish, typically chosen to be between 250 and 350 Hz. Furthermore, oscillating giant cells continued to oscillate after the lateral lemniscus was severed (i.e., this was their only input). For this experiment the lateral lemniscus was severed with fine scissors just anterior to the lateral lemniscus crossing. No differences in the firing pattern of the giant cells were observed. Giant cells continued to oscillate even though the inputs were unsynchronized and mainly irregular. Thus giant cells can be identified as endogenous oscillators. To check the possibility that endogenous oscillation is an artifact of the in vitro preparation, in vivo recordings of giant cells, following the method as described in Carr et al. (1986a), were performed. At zero stimulus field many giant cells fired periodically (see also Fig. 10 in Carr et al., 1986a).

During the dissection, and in particular during the removal of tissues covering the lateral lemniscus, the spherical cells in the electrosensory lateral line lobe undergo more mechanical stress than do the giant cells in the torus. In particular, the electrosensory lateral line lobe was frequently removed, thus removing the spherical cell somata. Thus spherical cell axons in this preparation are less likely to oscillate spontaneously in vitro, although they may in vivo.

Phase locking and time ambiguity

Delivering a current pulse typically 100 μs long and of 3 mA amplitude through an electrode placed on the decussation of the lateral lemniscus stimulates many spherical cell axons simultaneously. An action potential of a spherical cell axon can be recorded in lamina 6, i.e., about 1000 μm away from the stimulation site, with a latency Δ after the stimulus pulse of typically a few hundred microseconds, measured to the beginning of the spike at high gain. The jitter in the latency decreases with increasing stimulus amplitude and thus, the spherical cell axons fire in greater synchronization with each other. Each giant cell receives a total of 12–15 synapses from three or four spherical cells on its soma (Carr et al., 1986a,b), thus allowing for three or four potentially distinct postsynoptic potentials (psps). In this preparation with synchronized stimulation, the giant cell soma integrates three or four simultaneous psps a few hundred microseconds after the stimulus has been delivered. The giant cell then fires a spike with a latency depending on the stimulus amplitude and on the phase in the cycle of the periodically firing giant cell at which the simultaneous psps occur. In the case of periodic stimulation, the spherical cell afferents and giant cells may phase lock to the stimulus, depending on the frequency and amplitude of the stimulus.

In Fig. 2 we show a typical case of phase locking of a cell (giant cell or spherical cell axon) in lamina 6 to a periodic stimulus. Depending on the stimulus interval, we find M:N phase locking with M spikes for N stimulus pulses, such as 1:1, 1:2, 1:4, 1:6. The latter case is not stable. For values of stimulus intervals between ranges of stable phase locking, the cell fires irregularly (data not shown). Other values of M up to 4:1 have been observed in other giant or spherical cells in lamina 6. Only the case of 1:1 phase locking is behaviorally relevant, because only in this case does the timing of each spike represent information about the timing of the electric field in the receptive field of that cell. Furthermore, only this particular frequency range corresponds to frequencies naturally encountered by the fish. To convey unambiguously the timing of a stimulus, a cell should fire one spike

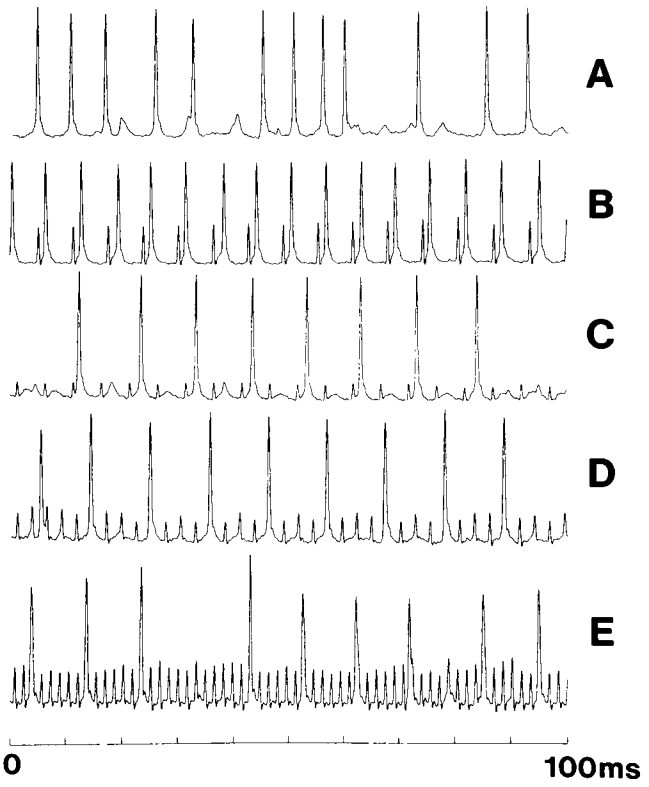

FIGURE 2 A typical case of M:N phase locking of a cell in lamina 6 (giant cell or spherical-cell afferent) to a periodic stimulus with stimulus interval τ. (*A*) no stimulus, the average cycle length T_o is 8.6 ms; (*B*) τ = 6.3 ms, 1:1 phase locking; (*C*) τ = 5.0 ms, 1:2 phase locking; (*D*) τ = 2.5 ms, 1:4 phase locking; (*E*) τ = 1.6 ms, 1:6 phase locking. The stimulus pulse is visualized by the stimulus artifact (small and short pulses).

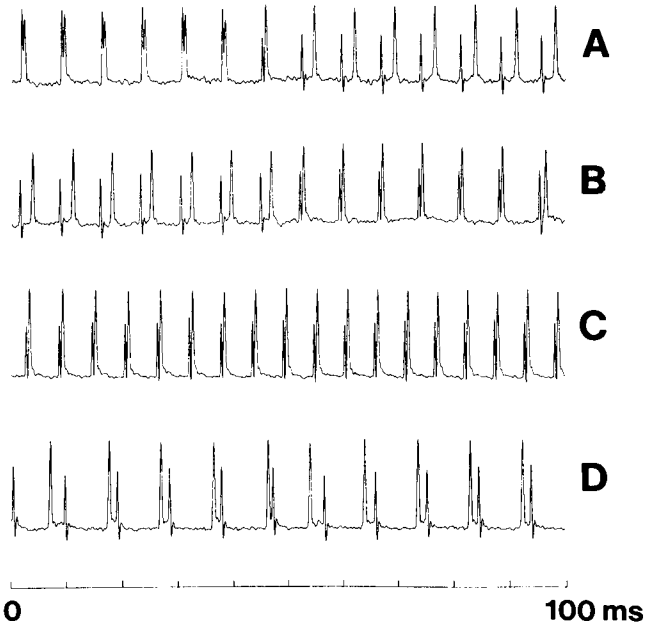

FIGURE 3 Phase locking with ambiguous representation of the timing of the stimulus pulse. (*A*) At τ = 7.3 ms the latency Δ jumps from Δ = 0.7 ms to 2.2 ms. (*B*) At τ = 7.3 ms the latency jumps from Δ = 2.2 ms to 0.7 ms. (*C*) At τ = 5 ms the latency is Δ = 1.0 ms. (*D*) At τ = 9 ms the latency is Δ = 8 ms.

with constant latency after the stimulus. Surprisingly, however, we encountered a few cells that in some stimulus frequency ranges showed latencies jumping between two values at a constant stimulus interval (Fig. 3, *A* and *B*), thus rendering the timing of the spike an ambiguous representation of the timing of the stimulus. To the author's knowledge, such latency jumps have not been reported previously. Furthermore, in some cases the latency of the spike was found to be a function of the stimulus frequency, as shown in Fig. 3, *C* and *D*.

Phase resetting and phase locking

If a stimulus pulse delivered in one cycle does not effect the next cycle, phase locking of an endogenous oscillator to a periodic stimulus with interval τ is related to the effect of single stimulus pulses in the following way (Perkel et al., 1964; Guevara et al., 1981) (Fig. 4). Let the phase ϕ be the time since the last spike, and let there be only one stimulus pulse between two successive spikes. The first stimulus hits the oscillator at phase ϕ_i and changes the cycle length to $T(\phi_i)$. After the interval τ the second stimulus hits the oscillator at the phase (see Fig. 4) $\phi_{i+1} = \phi_i + \tau - T(\phi_i)$. If the jitter in the cycle length of the endogenous oscillator is much smaller than the effect of the single stimulus pulse on the cycle length, the function $T(\phi)$ can be measured with single stimulus pulses placed at different phases and spaced sufficiently far apart so as not to disturb subsequent measurements. Once $T(\phi)$ is known, the previous equation can be iterated for a given τ and initial phase ϕ_o. If the sequence of phases converges, we find $\phi_{i+1} = \phi_i = \phi$, and from the previous equation we obtain $\tau = T(\phi)$. The range of values of $T(\phi)$ as determined by single-pulse stimulation are not all necessarily attainable during 1:1 phase locking. This is because one must also consider the question of stability. Stable 1:1 phase locking will result only when the slope of the $T(\phi)$ curve lies between 0 and 2 (Moore et al., 1963; Perkel et al., 1964). In other words, with the information

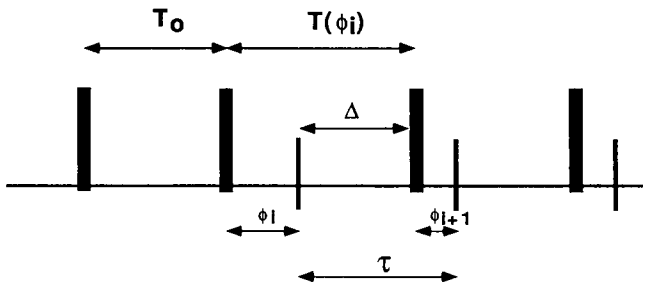

FIGURE 4 Diagrammatic representation of phase locking of a periodically firing cell (large bars, spikes) with cycle length T_o to a periodic stimulus (small bars, stimulus pulses) with interval τ. The first stimulus pulse is delivered at the phase ϕ_i, a spike occurs at the latency Δ, and the perturbed cycle length is $T(\phi_i)$. Subsequently, the second stimulus pulse hits the cell at the phase ϕ_{i+1}.

from single-stimulus experiments we can estimate for periodic stimulation a) the range of stimulus intervals and b) the phase (locking phase) at which phase locking occurs. For simplicity we assume that the jitter inherent in the cycle length of the endogenous oscillator renders the iteration redundant, i.e., if the endogenous oscillator phase locks to the periodic stimulus, it does so independently of the initial phase ϕ_0. In other words, from the equation $\tau = T(\phi)$ we can read the τ's and ϕ's at which phase locking occurs directly without performing the iteration.

Perturbed cycle lengths $T(\phi)$ were measured for 17 cells, of which four were labeled and identified as giant cells. Perhaps because of the mechanical stress caused by the dissection (see above), spherical cells tended to fire more irregularly, and thus, because of the preselection of periodically firing cells, the unlabeled cells were also likely to be giant cells. The curves $T(\phi)$ were measured as follows. Whenever a periodically firing cell with sufficiently low jitter was encountered, single pulses were delivered at 100-ms intervals. Because the stimulus interval was about 20 times larger than the average natural cycle length, it was reasonable to assume that a measurement was not affected by the previous stimulus pulse. Furthermore, no effects of the stimulus pulse on the cycle length of the next cycle could be detected. Because of the jitter inherent in the natural cycle length, the stimulus pulse hits the cell in a nearly random sequence of phases and, after a few seconds, the whole phase range has been scanned evenly. Then periodic stimuli were delivered at various stimulus frequencies, and the cell's response, i.e., either irregular firing or phase locking, was recorded. Finally, if possible, the cell was filled with neurobiotin for later identification. The perturbed cycle length $T(\phi)$ was then measured and plotted as the function of the phase ϕ at which the stimulus pulse was delivered.

In Fig. 5, we show data from a labeled giant cell for two

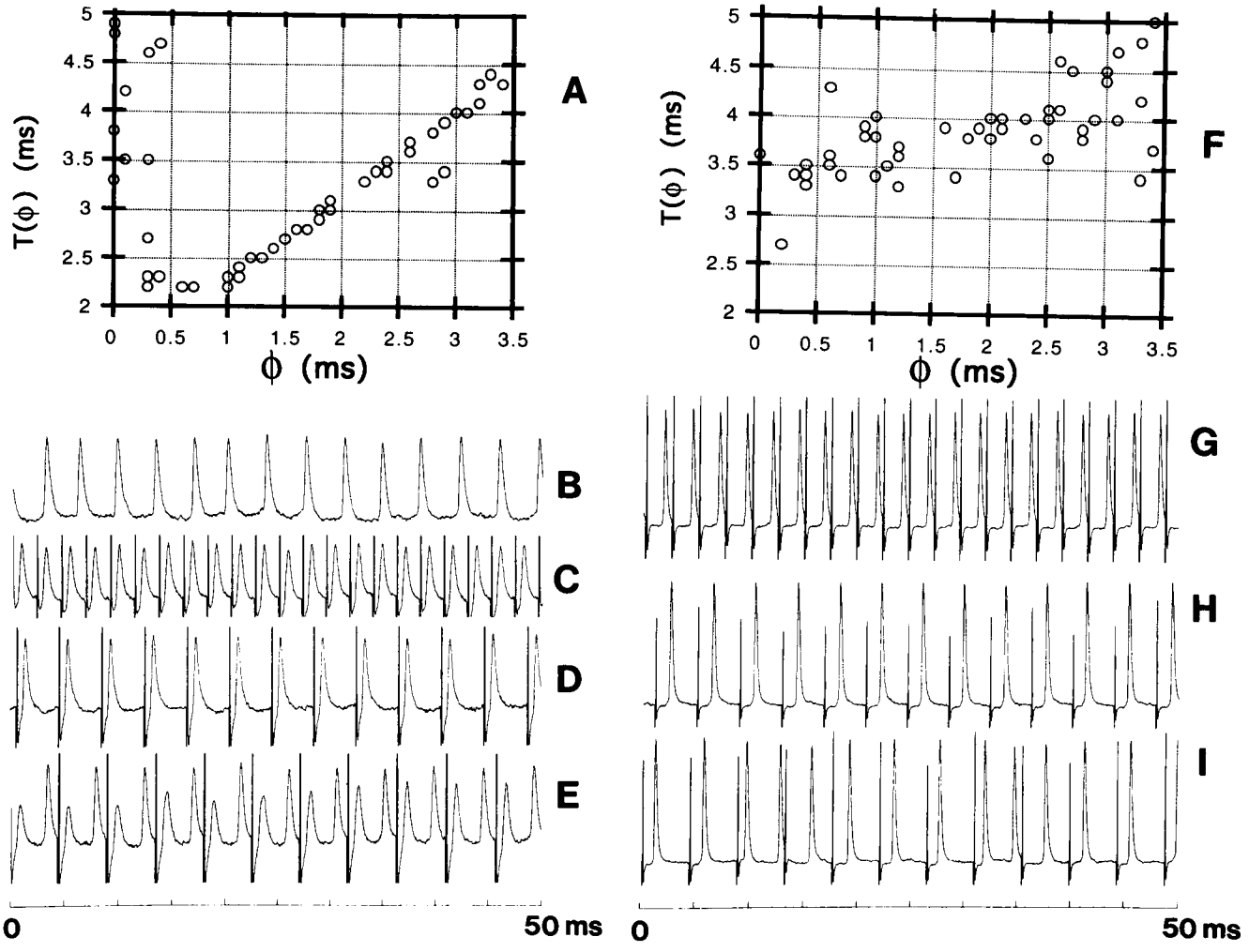

FIGURE 5 Single-pulse and periodic stimulation of a labeled giant cell at two different stimulus amplitudes, 5 mA (*A–E*) and 2.5 mA (*F–I*). (*A*) The perturbed cycle length $T(\phi)$, measured with single pulses, is plotted as a function of the phase ϕ, at which the pulse was delivered. (*B*) Without stimulus, the average cycle length is T_0 = 3.5 ms. (*C*) Periodic stimulation, τ = 2.3 ms, 1:1 phase locking at ϕ = 1.3 ms. (*D*) τ = 3.9 ms, ϕ = 3.1 ms. (*E*) τ = 4.5 ms, 2:1 phase locking. (*F*) $T(\phi)$ curve from single-pulse experiments. (*G*) Periodic stimulation, τ = 2.4 ms, ϕ = 0.6 ms. (*H*) τ = 3.9 ms, ϕ = 2.5 ms. (*I*) τ = 4.4 ms, irregular response.

different stimulus amplitudes, 5 mA (Fig. 5, *A–E*) and 2.5 mA (Fig. 5, *F–I*). The average free running cycle length T_o of this cell is 3.5 ms (Fig. 5 *B*). At the stimulus amplitude of 5 mA the $T(\phi)$ curve displays two distinct ranges. For $\phi < 1$ ms, the stimulus enters the refractory period of the giant cell. The spike amplitude decreases linearly to zero with decreasing ϕ over a few hundred microseconds, and the first spike after the stimulus occurs at about T_o (with large jitter, however). For the phase ϕ between 1 and 3.5 ms, the $T(\phi)$ curve is linear, and by fitting a straight line through the data points, we estimate the range covered by $T(\phi)$ to be between approximately 2.3 and 4.4 ms. The slope of the $T(\phi)$ curve is approximately 0.9. Spikes are fired at a latency of approximately 1 ms after the stimulus pulses. However, because the slope of $T(\phi)$ is less than 1, the latency decreases slightly with increasing phase at which the stimulus pulse was delivered. For comparison, at a phase-independent latency the $T(\phi)$ curve would follow $T(\phi) = \phi + \Delta$. From this curve (Fig. 5 *A*), we estimate, via $\tau = T(\phi)$ for periodic stimulation, a) there is 1:1 phase locking for the stimulus interval between approximately 2.3 and 4.4 ms and b) the spike occurs about 1 ms after the stimulus. These estimates from single-stimulus pulse experiments are in close agreement with the data from periodic stimulation (Fig. 5, *C–E*).

At the lower stimulus amplitude of 2.5 mA, the $T(\phi)$ curve is more jittery and almost flat (Fig. 5 *F*). Above the refractory period of $\phi = 1$ ms the $T(\phi)$ curve covers a range between approximately 3.5 and 4.5 ms, estimated by fitting a straight line through the data points. Thus, at this stimulus amplitude we estimate for periodic stimulation 1:1 phase locking for the stimulus interval τ between approximately 3.5 and 4.5 ms (Fig. 5 *F*). Surprisingly, phase locking to a periodic stimulus occurs between $\tau = 2.4$ and 3.9 ms (Fig. 5, *G* and *H*), i.e., for values of the stimulus interval that are beyond the range estimated from the single-pulse stimulation. The locking phases are different from those of the large-stimulus amplitude case. Although the stimulus amplitude is low, it is still well above threshold, as may be verified by the reliable phase locking of the cell to a periodic stimulus at the same stimulus amplitude. The inconsistency between the estimate from the single-pulse stimulation and the response to periodic stimulation suggests that the assumption (see first sentence in this subsection) that the response to a stimulus is simply dependent on the time since the last action potential does not hold at this stimulus amplitude. In other words, effects secondary to the stimulation may lead to a response to periodic stimulation different from the estimate from single-pulse stimulation.

An almost flat and jittery $T(\phi)$ curve was measured in other giant cells at low stimulus amplitude, such as the one shown in Fig. 6 *A*. As estimated from the single-pulse experiment, this cell 1:1 phase locks to a periodic stimulus at a stimulus interval near 4.1 ms. However, because of the large jitter compared to the almost flat $T(\phi)$ curve, the locking phase cannot be predicted from this single-pulse experiment. In fact, the large jitter compared to the almost flat $T(\phi)$ curve suggests either spike-to-spike variations in

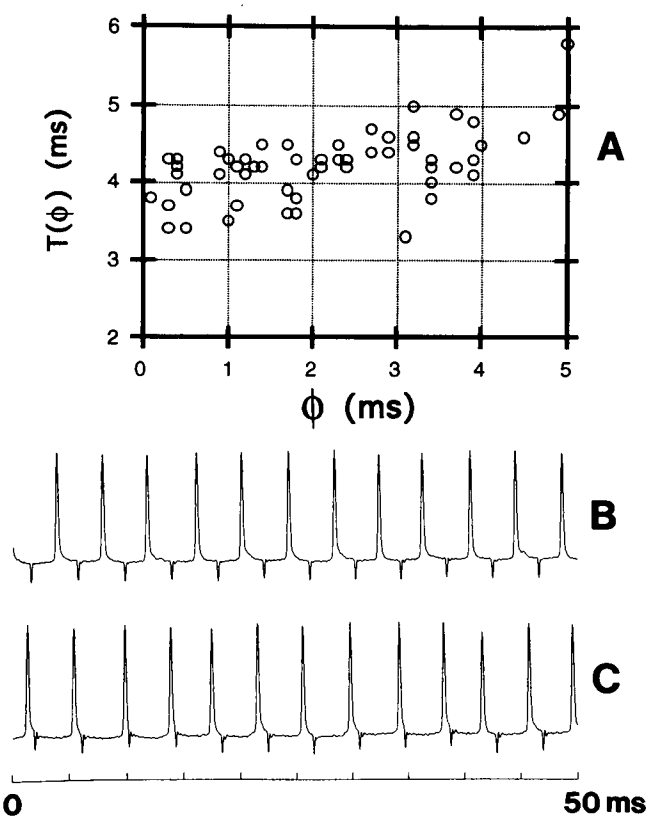

FIGURE 6 Single-pulse and periodic stimulation of a labeled giant cell at low stimulus amplitude. (*A*) $T(\phi)$ curve from single-pulse experiments. (*B*) Periodic stimulation with $\tau = 4.1$ ms, $\phi = 1.8$ ms. (*C*) Periodic stimulation with $\tau = 4.1$ ms, $\phi = 0.8$ ms.

the locking phase or the coexistence of stable 1:1 phase locking solutions with different locking phases. In the latter case, the solution chosen may depend on the initial condition or, because of the noise, the cell may spontaneously jump between different solutions. It turned out that in response to periodic stimulation with stimulus interval $\tau = 4.1$ ms, the cell 1:1 phase locked, as predicted by the single-pulse experiment, and the cell jumped spontaneously between two solutions with different locking phases of $\phi = 1.8$ ms (Fig. 6 *B*) and $\phi = 0.8$ ms (Fig. 6 *C*). Thus a jittery and almost flat $T(\phi)$ curve is consistent with the time ambiguity due to the cell jumping spontaneously between solutions with different latencies at constant stimulus frequency (see Fig. 3, *A* and *B*).

From neural oscillators in other systems (Winfree, 1977; Jalife and Antzelevitch, 1979; Guevara et al., 1981; Glass and Winfree, 1984), it is well known that the $T(\phi)$ curve may have an apparent discontinuity depending on the stimulus amplitude. An example of such a curve is shown in Fig. 7 *A*. Although we were not able to fill this cell, it is likely to be a giant cell (see above). The $T(\phi)$ curve shows two distinct ranges. For $T(\phi)$ between approximately 3.1 and 4.6 ms, $T(\phi)$ is linear, and by fitting a straight line through the data points we find the slope of the $T(\phi)$ curve to be approximately 0.9. Spikes are fired at a latency of approx-

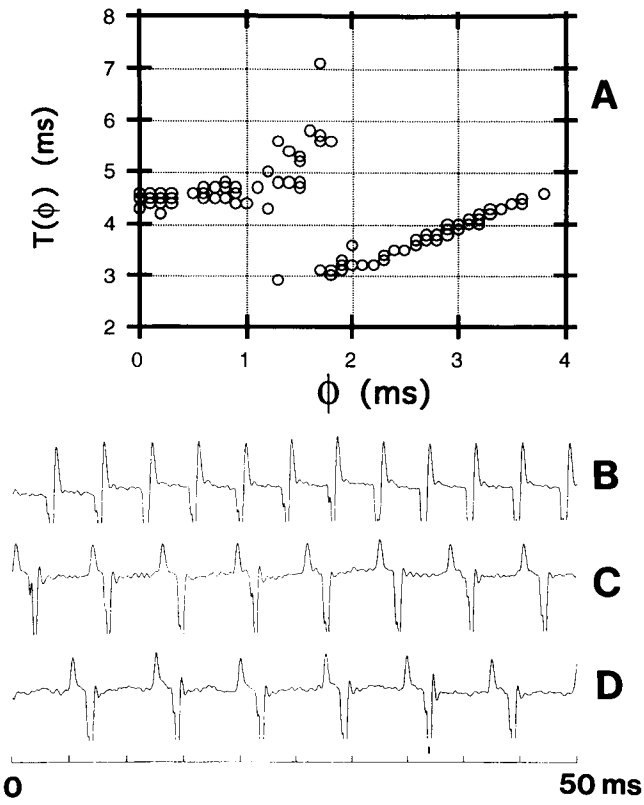

FIGURE 7 Single-pulse and periodic stimulation of a cell in lamina 6 (giant cell or spherical cell afferent). (*A*) $T(\phi)$ curve from single pulse experiments. (*B*) Periodic stimulation, $\tau = 4.1$ ms, $\phi = 3.2$ ms. (*C*) $\tau = 6.4$ ms, $\phi = 1.4$ ms. (*D*) $\tau = 7.4$ ms, $\phi = 1.5$ ms.

imately $\Delta = 1$ ms after the stimulus pulse. However, because the slope of $T(\phi)$ is less than 1, the latency shows a weak dependence on the phase at which the stimulus pulse was delivered (compare Fig. 5 *A*). Thus from the single-pulse experiments we estimate, via $\tau = T(\phi)$, 1:1 phase locking to occur at a stimulus interval between approximately 3.1 and 4.6 ms with a nearly constant latency of 1 ms. The response (Fig. 7 *B*) to a periodic stimulus of interval $\tau = 4.1$ ms is consistent with this estimate. For $T(\phi)$ above 5 ms the $T(\phi)$ curve is not linear and is more jittery. In particular, the slope of the $T(\phi)$ curve in this range is larger than 2. Because 1:1 phase locking will be stable only when the slope of the $T(\phi)$ curve lies between 0 and 2 (Moore et al., 1963; Perkel et al., 1964), we do not expect stable 1:1 phase locking for stimulus intervals larger than 5 ms. Surprisingly, the cell responded to periodic stimulation with stimulus intervals of $\tau = 6.4$ ms and $\tau = 7.4$ ms (Fig. 7, *C* and *D*) with 1:1 phase locking with locking phases consistent with the $T(\phi)$ curve. However, 1:1 phase locking was maintained over only a few seconds and was interrupted spontaneously by stretches of irregular firing.

Inhibition

Complete inhibition of periodically firing cells with a) a single stimulus pulse delivered at a specific phase (Jalife and Antzelevitch, 1979; Guttman et al., 1980) or b) periodic stimulation at certain frequencies (Perkel et al., 1964) has been reported. In a few cases, complete inhibition during periodic stimulation was found in this preparation. The clearest example is shown in Fig. 8. This cell was firing irregularly and phase locked to a periodic stimulus with a stimulus interval near 6 ms. This cell could be switched from phase locking to silence and vice versa by changing the stimulus interval by a few hundred microseconds. On average, phase locking occurred at $\tau = 6.0$ ms and inhibition at $\tau = 5.7$ ms. The value of the stimulus interval at which the transition occurred showed hysteresis.

Post-stimulus periodicity

In some cases an irregularly firing cell continued to fire periodically after phase locking to a periodic stimulus. A clear example of this effect is shown in Fig. 9. An irregularly firing cell phase locks to a strong periodic stimulus with a stimulus interval of 3.8 ms at a latency of 1 ms. After the periodic stimulus is stopped, the cell continues to fire

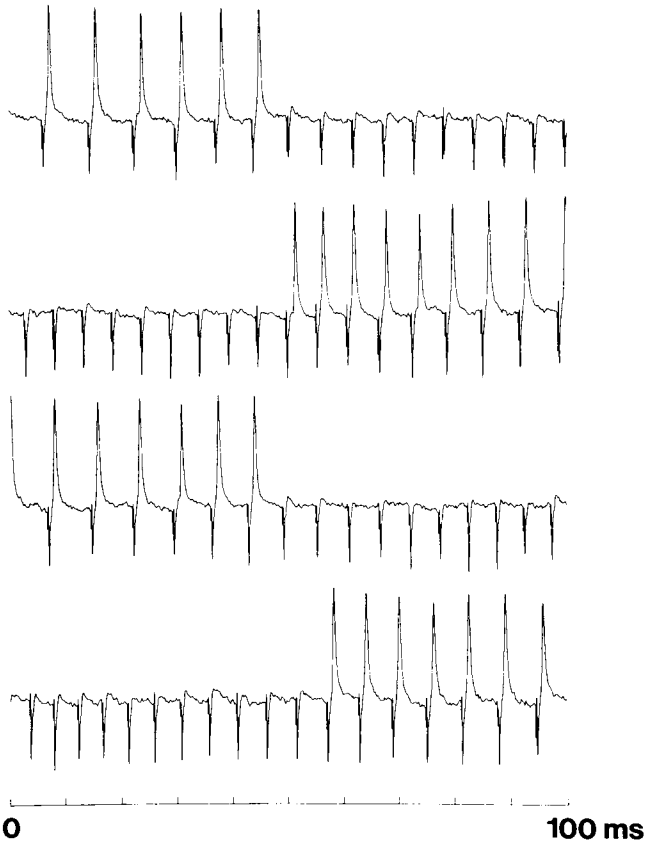

FIGURE 8 Phase locking and complete inhibition of a periodically firing cell (giant cell or spherical cell afferent) due to periodic stimulation. Transition from phase locking to inhibition occurred when changing the stimulus interval τ from 6.4 to 6.0 (trace 1), 6.5 to 6.0 (trace 3), 5.6 to 5.1, 5.5 to 5.3, 6.1 to 5.7, 5.3 to 5.1, 6.3 to 6.1, 5.7 to 5.4, and 5.5 to 5.3 ms. Transition from inhibition to phase locking occurred when changing the stimulus interval τ from 5.3 to 5.4 (trace 2), 5.6 to 5.8 (trace 4), 6.6 to 6.8, 5.7 to 6.0, 5.6 to 5.8, 6.1 to 6.2, 6.1 to 6.2, 6.0 to 6.2, and 5.9 to 6.0 ms.

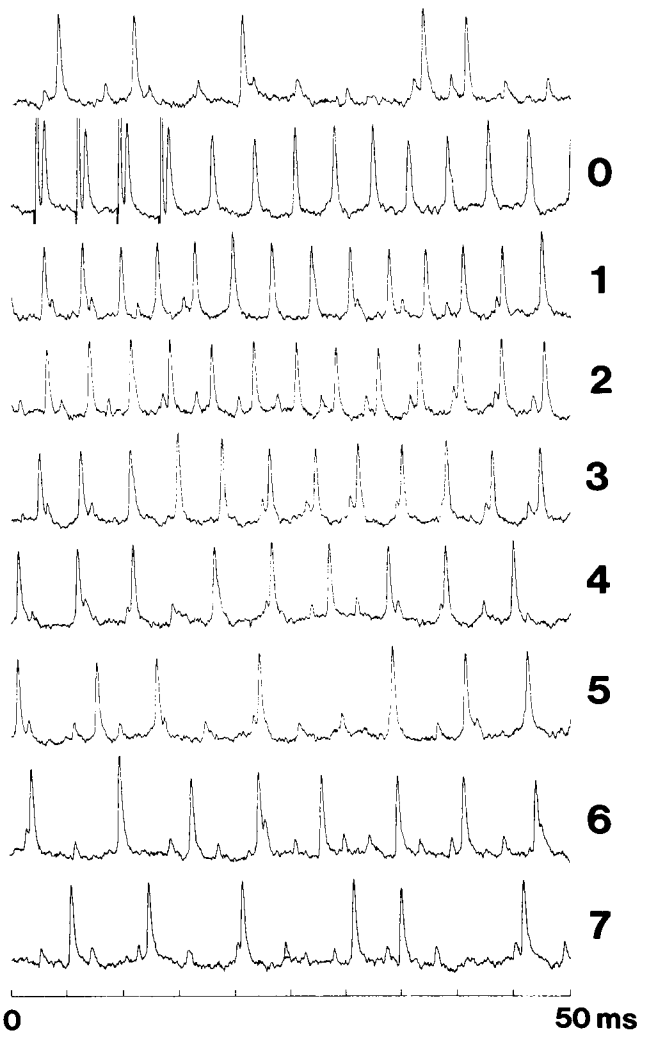

FIGURE 9 An example of post-stimulus periodicity. An irregularly firing cell (giant cell or spherical cell afferent) (first row) phase locks to a periodic stimulus with τ = 3.8 ms. After 2 s the stimulus is switched off (second row, 0 s). The following rows show traces of the firing pattern in intervals of 1 s after the stimulus has been switched off.

periodically with the same interval for about 3 s, then the cycle length increases and the cell falls back into its irregular firing pattern after about 6 s.

Bursting cells

Bursting spherical and giant cells are an artifact of the in vitro preparation, i.e., they have rarely been encountered in vivo. The occurrence of bursting spherical and giant cells in vitro varied greatly from preparation to preparation, from no bursting cells at all to most cells bursting. A perfectly spontaneously oscillating cell can be turned into a bursting cell by stopping saline and oxygen flow for more than 30 min, thus indicating that bursting is a sign of unnatural environment or bad health. However, for completeness their response to electrical stimulation of the lateral lemniscus is reported. A typical cell and stimulus amplitude are shown in Fig. 10. In the silent state, the cell fires one spike (or a train of spikes) with constant latency of about 2 ms after a single stimulus and phase locks to a periodic stimulus with an interval of 10 ms (Fig. 10, *A* and *B*). In the firing state, however, the cell largely ignores single stimuli and phase locks to a periodic stimulus, but only with larger jitter (Fig. 10 *C*).

DISCUSSION

Endogenous oscillators and post-stimulus periodicity

Although in vivo it is possible to record from periodically firing giant cells at zero electric field, never have all inputs to the giant cells been removed *in vivo*. *In vitro*, however, the lateral lemniscus can be cut and giant cells continue to fire periodically. Even if spherical cell axons had some regularity in their firing pattern, without a common stimulus they are not synchronized. It is not obvious how the unsynchronized and mostly irregular inputs to a giant cell could drive the cell into an oscillation close to the electric organ discharge frequency, and thus they are not believed to be the cause of the observed spontaneous giant cell oscillation. Thus it is concluded that giant cells are endogenous oscil-

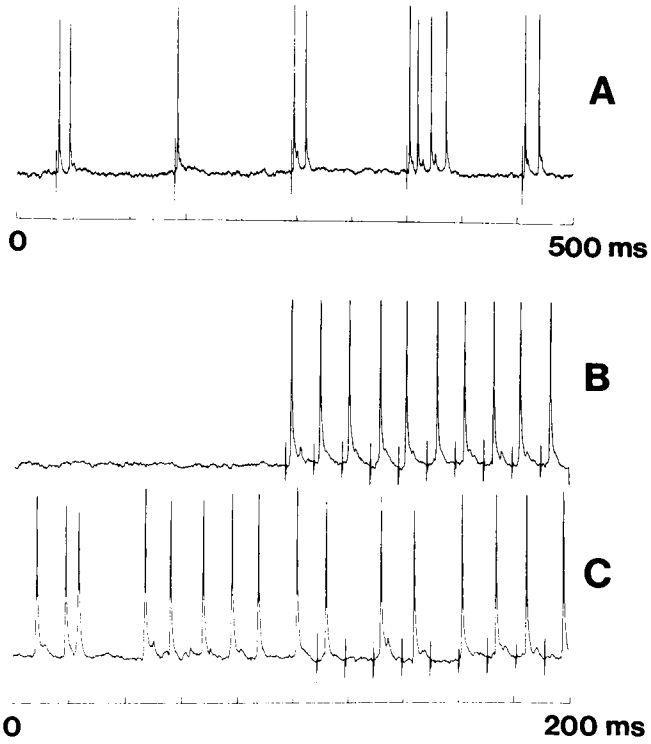

FIGURE 10 Response of a bursting cell (giant cell or spherical cell afferent) to electrical stimulation. (*A*) In its silent state the cell responds to single stimulus pulses delivered at 100 ms intervals with one spike or a train of spikes at a latency of approximately 2 ms after the stimulus pulse. (*B*) In its silent state the cell phase locks to periodic stimulation with τ = 10 ms. (*C*) In its firing state the cell phase locks to periodic stimulation with τ = 10 ms, but only intermittently and only with large jitter.

lators. Although it is possible that T-type afferents and spherical cells are endogenous oscillators as well, these cells were not subject of this study, because of technical difficulties in the stimulus delivery.

The occurrence of endogenous oscillators in this sensory pathway raises two questions:

1) What is the ionic basis of the oscillations? Giant cell somata have no dendrites and thus, because of the possibility of space clamping, are highly suitable for voltage clamp analysis. However, a) because of the small density of somata they are difficult to encounter and (b) it is likely that most of the current would leak through the gap junction connections to the inputs and through the thick axon. Recently, neurons from electric fish have been grown in culture (Turner et al., 1993), and thus, in the future, it may be possible to do voltage clamp analysis on cultured giant cell somata.

2) What is the functional advantage of endogenous oscillators, as opposed to silent or randomly firing cells, that follow their synaptic inputs? Linear oscillators have resonance frequencies, but neuronal oscillators are nonlinear oscillators and, thus, are much more complex. Aggregates of embryonic chick heart cells (Glass et al., 1987) as well as integrate and fire models (Glass and Belair, 1986) 1:1 phase lock to periodic stimulation with a small stimulus amplitude, as long as the stimulus frequency is close to the intrinsic frequency of the system. Within limits, the range of stimulus frequencies at which 1:1 phase locking occurs, as well as the stability of phase locking, increases with increasing stimulus amplitude. Also, it has been demonstrated that endogenous oscillators may phase shift (Jalife and Antzelevitch, 1979) or phase lock (Sano et al., 1978) to subthreshold stimuli. Thus, endogenous oscillators with an intrinsic frequency within the range of the stimulus frequencies have the functional advantage of 1:1 phase locking to the periodic stimulus, even at low stimulus amplitude.

It is possible that giant cells may become endogenous oscillators, tuned to the fish's electric organ discharge frequency, because of the periodic input. Activity shaped intrinsic electrical properties of neurons have been the subject of recent studies (LeMasson et al., 1993; Turrigiano et al., 1994). This speculation is supported by the observation that an irregularly firing cell phase locked to periodic stimulation and continued to fire periodically for several seconds after the stimulus had been switched off (post-stimulus periodicity, Fig. 9).

Phase locking

The response of giant cells to periodic stimulation showed both 1:N (N = 1 to 6) and M:1 (M = 1 to 4) phase locking (M spikes per N stimulus pulses). Theoretical studies of periodically forced van der Pol oscillators (Hayashi, 1964) as well as integrate and fire models (Glass and Belair, 1986) show that M:N phase locking with larger values of M and N is possible, but these occupy smaller areas in the frequency-amplitude space and thus, in the experiment, may be overlooked or obscured by noise.

Phase resetting and timing ambiguities

With single-pulse synaptic inputs the $T(\phi)$ curve was measured at various stimulus amplitudes. At low stimulus amplitude, the $T(\phi)$ curve is almost flat and jittery. At this amplitude, the cell may phase lock to periodic stimulation with a stimulus interval near the free cycle length, but the cell may jump between different solutions with different locking phases, thus rendering the timing of the post-synaptic spike an ambiguous representation of the timing of the pre-synaptic spikes. At larger stimulus amplitude, the $T(\phi)$ curve increases nonlinearly with ϕ until, at a certain phase, it decreases over a narrow phase range to a value below T_o and then, for larger ϕ, the cell fires with an almost constant latency Δ after the stimulus. At this stimulus amplitude, the cell phase locks to a periodic stimulus with stimulus intervals in the range covered by $T(\phi)$. For $\tau > T_o$, however, 1:1 phase locking is not stable, and the latency of the post-synaptic spike depends on the stimulus frequency. Thus its timing is an ambiguous representation of the timing of the pre-synaptic spike. At even larger stimulus amplitudes, the phase at which the jump in $T(\phi)$ occurs is shifted into the refractory period, and for $\phi > 1$ ms the $T(\phi)$ curve is linear with almost constant latency Δ. The cell 1:1 phase locks to a periodic stimulus with interval τ in the range covered by $T(\phi)$. At this amplitude the latency Δ is almost constant and the timing of the post-synaptic spike is an unambiguous representation of the timing of the pre-synaptic spike for all values of τ in the range covered by $T(\phi)$. Similar $T(\phi)$ curves have been measured on other systems, such as Purkinje fiber (Jalife and Moe, 1976), abdominal ganglia of *Aplysia* and tonic thoracic stretch receptors of *Procambarus* (Perkel et al., 1964), and cardiac pacemaker cells (Jalife and Antzelevitch, 1979; Guevara et al., 1981, 1986; Clay et al., 1984; Zeng et al., 1990).

Phase resetting and ionic currents

The ionic basis of phase resetting has been studied via modified Hodgkin-Huxley equations for the squid axon (Best, 1979; Guttman et al., 1980), the Purkinje fiber (Chay and Lee, 1984), heart cell aggregates (Clay et al., 1984) and a two-current excitable membrane model (Rinzel and Ermentrout, 1989). In general terms (Clay et al., 1984), a depolarizing current pulse applied at an early phase lengthens the cycle length by influencing the outward potassium current. A depolarizing current pulse applied at a late phase shortens the cycle length via a premature activation of the rapid inward sodium current. Whereas this general trend coincides with Fig. 7 *A*, in Fig. 5 *A* we see a lengthening of the cycle length at a late phase, $\phi > 2.5$ ms. This anomalous result is not easily explained on present ionic theory.

As the phase at which the stimulus pulse was delivered approached the refractory period (about 1 ms in these cells), the spike amplitude decreased linearly to zero, with decreasing phase over a few hundred microseconds. During a spike, most Na channels inactivate, the membrane is repolarized to the resting membrane potential, and the Na channels recover from inactivation (Hille, 1992). When a second spike is initiated during this recovery period, its amplitude is a function of the fraction of the Na channels that have recovered from inactivation at that time. Thus, Na channel inactivation and recovery explain qualitatively the drop in spike amplitude for spikes initiated at $\phi < 1$ ms. Information about the Na channel dynamics is required for a quantitative prediction.

Limit cycles and inhibition

Inhibition of neuronal oscillators by single excitatory stimuli is conveniently discussed with the concept of limit cycles (Glass and Mackey, 1988). A stable limit cycle is an oscillation (e.g., periodically firing cell) that is reestablished after a small perturbation (e.g., an injected current pulse). Depending on the precise nature of the oscillation the cell may have a stable steady state (silent) and a stable limit cycle within the same parameter range (e.g., value of DC current). In this case, the stable state chosen depends on whether this parameter range is approached from above or below, i.e., the system shows hysteresis (Guttman et al., 1980). Furthermore, it can be pushed with a single current pulse from the stable limit cycle into the basin of attraction of the stable steady state, thus stopping the oscillation (Jalife and Antzelevitch, 1979; Guttman et al., 1980). In addition, the oscillation can be halted by driving the parameter (e.g., DC current injection) into a range where only a stable steady state prevails (Guevara, 1987).

Now we extend this discussion to inhibition by periodic excitatory stimulation. For inhibition due to periodic excitation to occur, there has to be a particular phase Φ and stimulus interval τ for which the phase is reset by τ at each stimulus pulse. The amount by which a stimulus pulse, delivered at ϕ, resets the phase is measured by $T(\phi) - T_o$. Thus for inhibition to occur, there has to be a particular Φ and τ, such as $T(\Phi) - T_o = \tau$. This model is qualitatively different from inhibition due to single pulses, where a point on the limit cycle is pushed with a strong pulse into the basin of attraction of the steady state. To test the model one had to measure $T(\phi)$ with single pulses and check whether the equation $T(\phi) - T_o = \tau$ is valid for some Φ and τ. In the few cases where we observed inhibition, however, we were not able to measure $T(\phi)$, because the cells fired with too much jitter.

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